

OCCURRENCE OF MICROSCOPIC FUNGI AND MYCOTOXINS IN CONSERVED HIGH MOISTURE CORN FROM SLOVAKIA

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Abstract: Contamination by microscopic fungi and mycotoxins in high moisture corn (HMC) silages conserved by chemical additives was investigated. The samples were examined for the concentration and identification of microscopic fungi able to grow on Malt and Czapek-Dox agar and for mycotoxins content (deoxynivalenol, T-2 toxin, zearalenone and total aflatoxins, fumonisins, ochratoxins) by direct competitive enzyme-linked immunosorbent assays. The average fungal counts were $3.37 \pm 2.52 \log \text{cfu.g}^{-1}$ in control HMC silages, $2.91 \pm 0.51 \log \text{cfu.g}^{-1}$ in HMC silages treated by organic salts and inorganic salt, $3.62 \pm 1.46 \log \text{cfu.g}^{-1}$ in HMC ensiled with organic acids and $3.49 \pm 1.12 \log \text{cfu.g}^{-1}$ of HMC silages treated by organic acids along with organic salt. In this study, 740 isolates belonging to 10 fungal species representing 9 genera were recovered. The genera of microscopic fungi most frequently found in HMC were *Penicillium* (56.49%) and *Paecilomyces* (32.16%). T-2 toxin was the secondary metabolite with the highest concentration ranging from 179.13 ± 3.04 to $249.40 \pm 24.69 \mu\text{g.kg}^{-1}$, followed by deoxynivalenol and total fumonisins. The highest mean of deoxynivalenol level was $0.13 \pm 0.02 \text{mg.kg}^{-1}$ and concentration of total fumonisins ranged from 20.13 ± 2.53 to $90.33 \pm 10.35 \mu\text{g.kg}^{-1}$. This study indicated that application of chemical additives containing organic acids, organic salts and inorganic salt was sufficient to inhibit mycotoxins formation. The use of calcium formiate, sodium benzoate and sodium nitrite resulted in high hygienic quality of HMC silages and significantly reduced the concentration of zearalenone, deoxynivalenol and total ochratoxins and fumonisins.

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INTRODUCTION

In order to ensure good animal health and performance, it is essential to produce silages with high feed value and good hygienic quality. Apart from contamination of silages with undesired or even pathogenic microorganisms, e.g., *Clostridium tyrobutyricum*, *Clostridium botulinum*, *Listeria monocytogenes* and *Escherichia coli* O157 [10, 41], the occurrence of filamentous fungi (moulds) and their secondary

toxic metabolites (mycotoxins) attract considerable attention as potential causes for poor performance and health disorders in domestic livestock [5]. In comparison to cereal grains and proteinaceous feed materials, however, comprehensive knowledge of moulds and mycotoxins in silages and their effects on animal production is still lacking [38].

The term 'moulds' refers to a diverse group of microorganisms that are ubiquitous in nature and exist as saprophytes or plant pathogens. The genera *Fusarium*, *Alternaria*,

Cladosporium, *Penicillium* and *Aspergillus* are often considered as the most prominent feed-borne filamentous fungi [37].

The list of detected fungi could also be used by occupational disease and allergy specialists, taking into account that several varieties of *Penicillium*, *Alternaria*, *Cladosporium* and others fungi are considered to be hazardous moulds [15].

The aim of the study was to determine the occurrence of microscopic fungi and mycotoxins in high moisture corn from Slovakia conserved by chemical additives.

MATERIALS AND METHODS

Ensiling of HMC. The experiment was carried out at the Department of Animal Nutrition, Slovak University of Agriculture in Nitra, Slovak Republic. Harvested high moisture corn (HMC), grain hybrid Latizana, was immediately crushed by MURSKA 1000 HD grinder at 613.3 g.kg⁻¹ of dry matter. The experiment consisted of 4 variants. Three replicates per variant were ensiled. Control HMC silages were ensiled without additive (Variant C). The other HMC silages were ensiled with chemical additives. In the variant D, the chemical additive consisted of calcium formiate, sodium benzoate and sodium nitrite (powder form, applied at the rate of 3.5 g.kg⁻¹). Chemical additive used in variant E contained propionic acid and formic acid (liquid form, applied at the rate of 3.5 ml.kg⁻¹). HMC of variant F was conserved by liquid chemical additive (propionic acid, formic acid, benzoic acid and ammonium formate), and the application dose was 3.4 ml.kg⁻¹. All chemical additives were applied homogeneously. HMC matter was filled into plastic laboratory silos SJ 750 (cylindrical form, 50 dm³ of volume) and stored in an air conditioned laboratory at a conservation at temperature of 22°C. All silos were opened after 6 months of ensiling. HMC silages were sampled for occurrence of microscopic fungi and secondary metabolites (mycotoxins).

Isolation and morphological characterization of fungi.

For determination of fungi colony-forming units (CFU) was 1 g of sample soaked in 99 ml sterile tap-water containing 0.02% Tween 80 and then shaken for 30 min. Dilutions were prepared (from 10⁻¹ to 10⁻⁵) in sterile tap-water with 0.02% Tween 80, and 1 ml aliquots were inoculated on each of 3 plates of Czapek-Dox agar with streptomycin (to inhibit the bacterial growth). Petri dishes were inoculated using the spread-plate technique and incubated at 25°C. Total fungi CFU.g⁻¹ counts in samples were determined after 5 days of incubation. Malt agar (MA) and Czapek-Dox agar (CDA) were used to isolate and identify individual genera and species. Incubation was carried out at 25°C for 5–10 days. After isolation, or in the some cases monosporic isolation, individual species were identified on the basis of their macro- and micromorphology in accordance with other scientific reports [14, 18, 35].

Mycotoxin analysis. Mycotoxin content of the HMC silages was determined by direct competitive enzyme-linked immunosorbent assays (ELISA) [2]. A total of 40 samples of conserved high moisture corn were analyzed for 6 mycotoxins, including total aflatoxins (AFL), total fumonisins (FUM), total ochratoxins (OTA), zearalenone (ZON) deoxynivalenol (DON) and T-2 toxin (T-2). Samples of HMC silages for determination of mycotoxins were collected randomly from silage units (50 dm³). After that, samples were dried at 50°C (20 hours) and grounded to a fine powder. Extraction of samples was carried out in distilled water (DON), in methanol:water (70:30 v/v) for FUM, AFL, ZON and 50:50 (v/v) for T-2 and OTA. The Veratox quantitative test kits (Neogen, USA) were used and the ELISA procedure performed following the manufacturer's recommendations. The basis of the test is the antigen-antibody reaction. The wells in the microtiter plates were coated with antibodies to each mycotoxins. By adding standards of each mycotoxins or the sample solution the antibody binding sites were occupied in proportion to the concentration of each mycotoxin. Any remaining free binding sites were occupied in the next stage by enzyme labeled toxin (enzyme conjugate). Any unbound enzyme conjugate was then removed in a washing step. Enzyme substrate and chromogen were added to the wells and incubated. Bound enzyme conjugate converted the colourless chromogen into a blue product. The addition of the stop reagent resulted in a colour change from blue to yellow. Absorbance was determined using the microwell strip reader (Neogen, USA) at 650 nm. A calibration curve for the standards for each toxin dilution was plotted using a standard concentration against the percentage inhibition of the standard. For determination, each mycotoxins concentration was calculated by correlation coefficient from the following calibration curve: AFL 0.995 (r²=0.990), FUM 0.988 (r²=0.976), OTA 0.997 (r²=0.994), ZON 0.992 (r²=0.984), T-2 0.998 (r²=0.996), DON 0.997 (r²=0.994). Through the use of a microwell reader, the tests provided sample results in µg.kg⁻¹ for all mycotoxins except DON (mg.kg⁻¹).

Statistical analysis. Concentrations of mycotoxins were found in 3 repetitions for each sample of HMC silage at the same time. The results were statistically processed using one-factorial variance analysis (ANOVA) of SAS [36]. Means were separated using Fischer LSD multiple range test.

RESULTS AND DISCUSSION

Mould growth is determined by several environmental factors that markedly affect the composition of the mycoflora in feeds. Of particular importance are temperature, composition of the gas atmosphere, substrate properties, including moisture content and water activity (a_w), pH and chemical composition, as well as biotic factors (insects, vertebrates and other microorganisms) [22, 27].

Table 1. Total counts of microscopic fungi found in samples of high moisture corn silages on MA and CDA (log cfu.g⁻¹).

	\bar{x}	sd	v
C	3.37	2.52	74.55
D	2.91	0.51	17.61
E	3.62	1.46	40.46
F	3.49	1.12	32.13

MA – malt agar; CDA – Czapek-Dox agar; C – control variant (without additive); D – chemical additive consisting of calcium formiate, sodium benzoate, sodium nitrite; E – propionic acid and formic acid; F – propionic acid, formic acid, benzoic acid, ammonium formate; \bar{x} – mean; sd – standard deviation; v – coefficient of variation.

Fungal counts observed on malt extract and Czapek-Dox agar ranged from 0.47 log cfu.g⁻¹ to 4.87 of control samples, with an average 3.37 ± 2.52 log cfu.g⁻¹ of sample. Fungal counts observed on malt extract and Czapek-Dox agar ranged from 2.48 log cfu.g⁻¹ to 3.48 of samples with organic salts and inorganic salt, with an average 2.91 ± 0.51 log cfu.g⁻¹ of sample. Fungal counts observed on malt extract and Czapek-Dox agar ranged from 2.00 log cfu.g⁻¹ to 4.85 of samples with organic acids along with organic salt, with an average 3.62 ± 1.46 log cfu.g⁻¹ of sample. Fungal counts observed on malt extract and Czapek-Dox agar ranged from 2.48 log cfu.g⁻¹ to 4.85 of samples in variant F, with an average 3.49 ± 1.12 log cfu.g⁻¹ of sample (Tab. 1). No significantly lower number of microscopic fungi was found. Similar results concerning the fungal counts in silages were found in farm silages from northern Germany [4].

During this study 740 isolates belonging to 10 fungal species representing 9 genera were recovered. The incidence and total number of isolated fungi in Slovakian silages are presented in Table 2. The genera of microscopic fungi most frequently observed in HMC were *Penicillium* (56.49%) and *Paecilomyces* (32.16%). In a survey in France and Italy in

3 successive years, Pelhate [30, 31] analyzed the mycoflora of 1,230 maize silage samples from commercial farms and isolated a non-exhaustive count of 70 fungal species. The study by Gedek *et al.* [12] included 260 farm silages in Germany and showed a high degree of contamination with *P. roqueforti* and *A. fumigatus*. These species were detected in 71.3 and 13.3% of the samples, respectively. *Mucor*, *Absidia*, *Rhizopus*, other *Penicillia* and *Aspergilli* as well as *Monascus*, *Byssochlamys* and *P. varioti* were less frequently isolated. Another investigation in the composition of the mycoflora of silages in southern Germany showed the prevalence of *P. roqueforti*, which was isolated from 27% of the samples. Species of minor incidence were *Rhizopus nigricans*, *A. fumigatus* and *M. rubber* [3].

The analytical results for mycotoxins (Tab. 3) indicate that samples of HMC silages were contaminated with almost all determined mycotoxins. After 6 months of storage, only DON was not detected in E variant of HMC (treated with propionic and formic acid) at the detection level mg.kg⁻¹. The most prevalent mycotoxin was T-2 toxin, followed by DON and FUM. T-2 toxin is associated with reduced feed consumption, loss in yield, gastroenteritis, intestinal haemorrhage, reduced reproductive performance and death [43]. T-2 toxin was detected at concentrations from 179.13 ± 3.04 (F) to 249.40 ± 24.69 (E). Differences in T-2 concentration observed between variants of HMC were not significant ($p > 0.05$). Higher T-2 values were observed in North Carolina for corn grain (569 ± 690 $\mu\text{g.kg}^{-1}$) [29].

Fumonisin affect animals in different ways by interfering with sphingolipid metabolism [24]. They cause leukoencephalomalacia in horses, pulmonary edema in swine, and have hepatotoxic and carcinogenic effects [33]. The HMC of variant D had significantly ($p < 0.05$) the lowest content of FUM (20.13 ± 2.53 $\mu\text{g.kg}^{-1}$). The highest FUM concentration was identified in variant E of HMC treated

Table 2. Fungi recovered from Slovakian high moisture corn silages.

Fungi	C	D	E	F	Total	
					Isolates	Frequency (%)
<i>Acremonium</i> sp.	1	–	1	–	2	0.27
<i>Aspergillus flavus</i> Link : Fr	6	2	2	2	12	1.62
<i>Fusarium</i> sp.	–	–	1	9	10	1.35
<i>Mycelia sterilia</i>	–	1	–	–	1	0.14
<i>Mucor circinelloides</i> v. Tiegh.	–	–	22	–	22	2.97
<i>Mucor racemosus</i> Fres	–	–	32	–	32	4.32
<i>Rhizopus stolonifer</i> (Ehrens. : Fr.) Vuill.	2	–	–	2	4	0.54
<i>Paecilomyces varioti</i> Bain.	–	–	29	209	238	32.16
<i>Penicillium</i> sp.	287	2	103	26	418	56.49
<i>Scopulariopsis</i> sp.	–	–	1	–	1	0.14
Total	296	5	191	248	740	100

C – control variant (without additive); D – chemical additive consisting of calcium formiate, sodium benzoate, sodium nitrite; E – propionic acid and formic acid; F – propionic acid, formic acid, benzoic acid, ammonium formate.

Table 3. Concentrations of mycotoxins in high moisture corn silages.

µg.kg ⁻¹	C			D			E			F		
	\bar{x}	sd	v	\bar{x}	sd	v	\bar{x}	sd	v	\bar{x}	sd	v
ZON	38.97	4.68	12.01	33.07 ^a	3.37	10.18	37.83	5.08	13.42	56.33 ^a	3.42	6.08
T-2	232.17	21.67	9.33	216.13	27.51	12.73	249.40	24.69	9.90	179.13	3.04	1.70
AFL	2.47	0.21	8.59	2.00	0.20	10.18	2.70	0.34	12.57	1.80	0.10	5.66
FUM	70.20 ^a	7.24	10.31	20.13 ^{abc}	2.53	12.58	90.33 ^{bd}	10.35	11.46	50.37 ^{cd}	4.06	8.06
OTA	2.33 ^{abc}	0.24	10.20	0.77 ^{ad}	0.09	11.80	1.37 ^b	0.18	13.04	1.23 ^{cd}	0.11	8.60
DON*	0.13 ^{ab}	0.02	12.76	0.07 ^a	0.01	14.14	nd	–	–	0.07 ^b	0.01	14.14

ZON – zearalenone; T-2 – T-2 toxin; AFL – total aflatoxins; FUM – total fumonisins; OTA – total ochratoxins; DON – deoxynivalenol; * mg.kg⁻¹; nd – non-detected; C – control variant (without additive); D – chemical additive consisting of calcium formiate, sodium benzoate, sodium nitrite, E – propionic acid and formic acid; F – propionic acid, formic acid, benzoic acid, ammonium formate; ^{a, b, c, d} – the values with identical superscripts in a row are significantly different at $p < 0.05$.

with organic acids (90.33 ± 10.35 µg.kg⁻¹). The HMC of variant E had a significantly higher FUM concentration than HMC other experimental treatments. Also, the study of Marin *et al.* [23] confirmed that concentrations of FUM were unaffected by treatment with a commercial antifungal preservative based on a mixture of propionates. Kedera *et al.* [16] detected little values of fumonisin B₁ in most of the samples of maize kernel from Kenya. Fumonisin B₁ contained 47% of the samples at levels above the detected limit 100 µg.kg⁻¹, but only 5% were above limit 1000 µg.kg⁻¹. In 2006, Golian *et al.* [13] reported a higher average concentration of FUM in comparison with our results. Deoxynivalenol is one of the most common mycotoxins found in grains. DON is associated with nausea, vomiting, diarrhea, weight loss and food refusal [34]. In Poland, Krysińska-Traczyk *et al.* [19] analysed the average concentration of DON in cereals of 0.3775 mg.kg⁻¹ and unacceptably high values (up to 927 mg.kg⁻¹) for DON were recorded for maize grain and cobs [32]. *Fusarium* species isolated from maize in Minnesota produced the DON in a range from 4–0.225 mg.kg⁻¹ [1] and analysis of corn samples collected in Wisconsin revealed an overall average concentration of total DON 0.2377 mg.kg⁻¹ [28]. In the present study, the samples of control HMC silages had the highest mean level of DON (0.13 ± 0.02 mg.kg⁻¹), while the samples of D and F variants contained the same mean levels (0.07 ± 0.01 mg.kg⁻¹).

The chemical additives treatment in variant D and F had a significant effect on DON concentration, compared with the untreated HMC silages.

Zearalenone is the cause of hyperestrogenism [40], vaginal and rectal prolapse [11], and abortions. The results of our observation resulted in ascertaining that contamination by ZON (range from 33.07 ± 3.37 (D) to 56.33 ± 3.42 (F) µg.kg⁻¹) was similar to the study by Nuryono *et al.* [26] and Kim *et al.* [17], otherwise lower than in an investigation by Whitlow and Hagler [44], and higher in comparison with results of Lepom *et al.* [21]. Application of chemical additives did not affect ($p > 0.05$) the concentration of ZON, but the results of Paster *et al.* [29] showed that NH₄Cl totally

inhibited zearalenone production in corn grains. In our study, data showed that concentrations of ZON in treated HMC silages were not significantly different in comparison with control. There were significant differences in ZON values only between variants D and F. Limited values for DON, ZON and FUM in maize and products derived from maize crops are 8, 2 and 60 mg.kg⁻¹, respectively. In none of the HMC silages analysed in the study was the EC guidance values for DON, ZON and FUM exceeded [9].

Aflatoxins are toxic and highly carcinogenic secondary metabolites [6]. Aflatoxins are associated with inappetance, ataxia, enlarged fatty livers, reduced feed efficiency and milk production, jaundice and decreased appetite [25]. The lowest contamination of high moisture corn samples was by AFL and OTA mycotoxins. No significantly lower content ($p > 0.05$) of AFL was found in HMC treated with organic salts and inorganic salt (D) and with organic acids along with organic salt (F) than in untreated HMC. Our results confirmed that aflatoxin production was reduced by benzoic acid and sodium benzoate [42]. Benzoates and their derivatives are used as antimicrobial agents. From all benzoate derivatives detected by Chipley and Uraih [7] the most effective were methyl benzoate and ethyl benzoate in reducing both mycelial growth and aflatoxin release by *A. flavus* and *A. parasiticus*. Ochratoxin A is nephrotoxin, liver toxin, immune suppressant, potent teratogen and carcinogen [20]. In our study, the content of OTA in all experimental variants of HMC silages was significantly lower ($p < 0.05$) than the maximum level (control). AFL was detected in concentrations ranged from 1.8 ± 0.10 (F) to 2.47 ± 0.21 µg.kg⁻¹ (C). Mycotoxin production from maize gluten and other maize products was evaluated by Scudamore *et al.* [39]. Aflatoxins were not found above the reporting limit ($1–5$ µg.kg⁻¹) in any sample, while ochratoxin A was detected only in two samples of maize gluten (2 µg.kg⁻¹). Our study demonstrated that the levels of OTA and AFL in samples of HMC silages were very low and levels did not exceed the recommended limits for cereals and products derived from cereals (250 and 20 µg.kg⁻¹, respectively) [8, 9].

CONCLUSIONS

Moulds and mycotoxins are common contaminants of forage crops and silages made from them in many areas of the world. They pose a potential health hazard to domestic livestock. Changes in environmental conditions from pre-ensiling through fermentation result in the establishment of a characteristic mycoflora, mainly represented by *Penicillium*, *Aspergillus* and *Fusarium* species. In addition to field-derived mycotoxins, the proliferation of these filamentous fungi upon subsequent exposure to air during feedout can result in further increase in the mycotoxin load of silages. Good management of the growing crop, the ensiling process and the unloading phase must be employed to minimize mould and mycotoxin contamination, and the use of antimycotic silage additives should be implemented as a strategic tool in silage making technology. Adhering to those principles will certainly reduce the risk of nutrient losses by fungal development and, even more importantly, will substantially counteract the impact of their toxic metabolites on animal health and performance.

Occurrence of observed mycotoxins was detected in all HMC silages except for DON in HMC silages with propionic and formic acid at the detection level mg.kg⁻¹. The results show that T-2 toxin was the secondary metabolite with the highest concentration. T-2 toxin was followed by *Fusarium* produced mycotoxins, DON and FUM. These data indicate that application of chemical additives can significantly reduce the concentration of mycotoxins in HMC silages. The addition of chemical additive consisting of calcium formiate, sodium benzoate and sodium nitrite inhibited mycotoxins formation the most effectively. The reason for the low production of mycotoxins after application of chemical additives is the subject for further investigation.

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